Analysis of Temperature-Sensitive Mutants Reveals New Genes Involved in the Courtship Song of Drosophila

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ABSTRACT

cacophony (*cac*), a mutation affecting the courtship song in *Drosophila melanogaster,* is revealed to cause temperature-sensitive (TS) abnormalities. When exposed to high temperatures (37°), *cac* flies show frequent convulsions and pronounced locomotor defects. This TS phenotype seems consistent with the idea that *cac* is a mutation in a calcium-channel gene; it maps to the same *X*-chromosomal locus that encodes the polypeptide comprising the α-1 subunit of this membrane protein. Analysis of the courtship song of some TS physiological mutants showed that *slowpoke* mutations, which affect a calcium-activated potassium channel, cause severe song abnormalities. Certain additional TS mutants, in particular $para^{ts1}$ and nap^{ts1}, exhibit subtler song defects. The results therefore suggest that genes involved in ion-channel function are a potential source of intraspecific genetic variation for song parameters, such as the number of cycles present in "pulses" of tone or the rate at which pulses are produced by the male's courtship wing vibrations. The implications of these findings from the perspective of interspecific lovesong variations in Drosophila are discussed.

DURING courtship, males of *Drosophila melanogaster* and of many other species vibrate their wings, producing a "lovesong" (Bennet-Clark and Ewing 1970). This has been implicated as a potential species recognition signal (*e.g.*, Kyriacou and Hall 1982, 1986). A few mutations affecting courtship song have been isolated (Hall 1994a). Among these, *cacophony (cac)* is particularly interesting, for example, from an evolutionary point of view. The song of *cac* males is characterized by longer interpulse-intervals (IPIs) and pulses that contain more cycles than normal (Schilcher 1977; Kulkarni and Hall 1987). These are two features that commonly show differences among Drosophila species (*e.g.*, Cowling and Burnet 1981; Ewing and Miyan 1986; Hoikkala and Lumme 1987).

cac maps to a locus on the *X*-chromosome that is also the site of *night-blind-A* (*nbA*) visual and *l(1)L13* lethal mutations. These genetic variants show a complex pattern of complementation. While the *l(1)L13* mutations fail to complement the song and visual defects of *cac* and *nbA*, respectively, *cac*/*nbA* flies are apparently normal (Kulkarni and Hall 1987). Recently, we cloned a gene encoding a new α-1 subunit of a voltage-sensitive calcium channel, named Dmca1A (Smith *et al.* 1996; Peixoto *et al.* 1997), which maps to the *cac* locus. Previously, only one other voltage-sensitive calcium channel (Dmca1D) was known in Drosophila (Zheng *et al.* 1995), but no behavioral defects have as yet been associated with variations at the autosomal locus encoding Dmca1D.

Ion channels are a diverse class of transmembrane proteins involved in a plethora of cellular phenomena (Hille 1992). Voltage-gated calcium channels, for example, are usually divided into six different classes according to their electrophysiological characteristics, pharmacology, sequence similarities, and tissue distribution (Stea *et al.* 1995). They are involved in many important processes, such as neurotransmitter release and muscular contraction (McCleskey 1994). Mutations affecting ion-channel function in *D. melanogaster* are often associated with temperature sensitivity, paralysis, and related phenotypes (Wu and Ganetzky 1992). Here we report that *cac* flies show a temperature-sensitive (TS) abnormality that can best be termed a convulsion. This phenotype is consistent with *cac* being a mutation in a calcium-channel gene. We also analyzed the song of additional TS mutants, including other ion-channel ones. The results indicate that mutations in genes encoding or affecting ion-channel function are a source of intraspecific variation for the Drosophila's lovesong.

MATERIALS AND METHODS

Basic fly handling and Drosophila strains used: Flies were reared on a sucrose-cornmeal-yeast-Tegosept medium in glass vials (the last ingredient is a mold inhibitor). Stocks were maintained in 12hr:12hr, light:dark (LD) cycles at 25° and

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70% relative humidity. Flies were collected and separated by sex as <1-day-old adults under CO_2 anesthesia.

The following stocks, involving genetic variations at the *X*-chromosomal *cac* locus, were used in tests of general locomotion and courtship song, except for certain heterozygous female types (see below): unmarked *cac*, *night-blind-A*^{EE171}, and *nbA*^{H18}. All three of these mutants had been separately backcrossed seven times to an attached-*X* stock: *C(1)DX, y f*. To generate hemizygous mutant females for testing of general locomotion, males from the above stocks were crossed to females from a *In(1)FM7c/Df(1)RC29 g* stock (*FM7c* is an *X*-chromosome balancer mutation, and *g* is an eye-color mutation). This cross yields heterozygotes carrying each one of the alleles over the deficiency *RC29*, which uncovers the mutant effects of *cac* and *nbA* alleles (Kulkarni and Hall 1987). Testing of *cac/RC29* females for nonsexually dimorphic phenotypes controlled (as did the backcrossing noted above) for genetic background effects of any recessive factors remaining on the *cac*-bearing *X*-chromosome that might contribute to the impairments in question (*e.g.*, Table 1). *cac*$^+$ control flies, and those used to generate certain *cac*$^+$-carrying heterozygotes, were derived from a Canton-*S* wild-type stock that had been backcrossed seven times to the attached-*X* stock indicated above.

The following TS mutants were subjected to the song analyses (see below): *seizure* (*sei*^{ts1}), *temperature-induced-paralysis-E* (*tipE*), *paralytic* (*para*^{ts1}), *no-action-potential* (*nap*^{ts1}), *slowpoke* (alleles: *slo*^{1} and *slo*^{2}), and *cysteine-string protein* (*csp*^{X1}) (Lindsley and Zimm 1992; Zinsmaier *et al.* 1994). All of these mutations are autosomal except *para*^{ts1}. sei^{ts1} is a mutation in a gene encoding a potassium channel of the *eag* subfamily, which is closely related to the HERG inwardly rectifying potassium channels (Titus *et al.* 1997; Wang *et al.* 1997). *sei*^{ts1} causes flies, when exposed to high temperatures, to exhibit uncontrolled flight activity followed by partial paralysis. *para*^{ts1}, *tipE*, and *nap*^{ts1} are mutations affecting sodium-channel function that cause flies to paralyze at high temperatures (reviewed in Wu and Ganetzky 1992); the *paralytic* locus encodes the α-1 subunit of a voltage-dependent sodium channel (Loughney *et al.* 1989). The *tipE* locus codes for a novel membrane protein that enhances the function of *para* sodium channels (Feng *et al.* 1995). *nap*^{ts1} is a gain-of-function allele of *mle*, a gene that is required for *X*-chromosome dosage compensation (Kernan *et al.* 1991), and it probably affects *para*-gene expression (*cf.* Wu and Ganetzky 1980). *slo*^{1} and *slo*^{2} are mutations in the *slowpoke* gene, which codes for a calcium-activated potassium channel (Atkinson *et al.* 1991; N. S. Atkinson, personal communication; B. Ganetzky, personal communication). Flies with mutations in this locus are uncoordinated and unable to climb at high temperatures. Even at lower temperatures they show abnormal locomotor behavior and diminished flight ability (Elkins *et al.* 1986). *slo*^{2} seems a more debilitating mutation in the sense that its homozygous stock was far more difficult to maintain at 25° than *slo*^{1}. The *cysteine-string protein* (*csp*) gene is involved in neurotransmitter release (Zinsmaier *et al.* 1994) and is thought to potentially interact with calcium channels (Mastrogiacomo *et al.* 1994). The *csp*^{X1} mutant allele causes TS paralysis and early death (Zinsmaier *et al.* 1994).

Some of these mutants were also analyzed in a *cac* background (see results). An attached-*X* stock was generated in which males were hemizygous for *para*^{ts1} by backcrossing (seven times) flies from the available homozygous stock to the same *C(1)DX, y f* stock used as above. The majority of recordings were carried out using males from a given attached-*X* stock, but males from the homozygous *para*^{ts1} stock were also recorded. The stocks containing *nap*^{ts1} and *csp*^{X1} were maintained, respectively, with *In(2LR)O, Cy* (*CyO*) and *In(3LR)TM3, Sb* (second- and third-chromosomal balancers). Homozygous stocks of the mutants *sei*^{ts1}, *tipE* (marked with *se*), *slo*^{1} (marked with *st*), and *slo*^{2} (*se* and *st* being eye-color mutants) led to males used in the song recordings. In the case of *slo*^{1} and *slo*^{2}, males from balanced stocks (using *In(3LR)TM2, Ubx*, and the *TM3* stock described above), produced using the original homozygous mutant strains, were also recorded. In Tables 3–5, some of the flies heterozygous for the third chromosome mutations *csp*^{X1}, *slo*^{1}, and *slo*^{2} also carried a balancer (*TM2* or *TM3*).

General behavioral tests: To quantify loss of body control in heated *cac* adults, the percentage of time that 20-day-old flies spent on their backs or sides, curling their abdomens, or spinning around was measured. The initial observations of these phenotypes were carried out by placing flies either over a hot plate brought to ~37° (see below) or inside an incubator (also at ~37°) with a glass door. To measure the percentages of time that the flies exhibited one of the three features of these convulsions, a plastic device with a circle of cylindrical chambers (each 10 mm diameter × 3 mm height) was prewarmed to ~37° (for the hot-plate application, the device was placed within a 25° temperature-constant room). One min after the animals were introduced into such chambers with the aid of an aspirator (hence no further anesthesia), they were observed and timed (one fly at a time) for 5 min. The timers involved a bank of electrical devices that accumulated time when switches attached to each timer were flicked on, then off again when the abdominal curling (say) ceased.

A genotypically more extensive version of testing for heat-induced convulsions also involved an adult-age component (Table 1). For this, one to three flies were observed at one time after placing them in a prewarmed mating wheel. The temperature on the surface of the hot-plate was raised to 38–39°, so that the top of the wheel of chambers was 35–36°. Both of these temperature values were measured with a thermometer whose probe lies flat on the surface in question. It was estimated that the temperature inside the chambers was ~37°. (As above, the hot-plate experiments leading to Table 1 were carried out inside a 25° room.) One min was allowed to elapse before the start of the observations, for which only the "on-their-backs-or-sides" phenotype was quantified as a percentage of the following 14 min after a 1-min interval, the subsequent 14 min of behavior was also quantified (Table 1).

Knock-out tests were performed in which eight flies (two from each of four genotypes) were aspirator-loaded into separate chambers of the wheel, which was kept initially at room temperature (25°). The wheel was then placed over the hot-plate at 46° (±0.5°) for 10-min trials to ascertain which flies were knocked out on the floor of the chamber within this period (Table 2).

Courtship song: Recordings and song analysis was carried out as described by Bernstein *et al.* (1992) and Villella and Hall (1996), using an INSECTAVOX (Gorczyca and Hall 1987) and LifeSong software (Bernstein *et al.* 1992). Courtships were recorded for ~5 min using a Sony Hi8 video camera (Sony, Parkridge, NJ). Only pulse song was examined in this report (courtship hums, or sine-song, being the other song, *e.g.*, Wheeler *et al.* 1989; Villella and Hall 1996). Usually, all the pulses of the song of a given fly are logged, that is, marked for storage in the relevant file using the computer as an event-recorder, while scanning the visual record of the song along with the video image of the flies' behavior. Logging of some songs extended for only 2 min, and more than 500 pulses were typically logged. Songs with less than 40 pulses were not included in the analysis. Four parameters of the flies' pulse song were measured: interpulse interval (IPI), Cycles-per-Pulse (CPP), amplitude, and intrapulse frequency (IPF) (*cf.* Wheeler *et al.* 1989; Bernstein *et al.* 1992). CPP

and IPF values can vary together among Drosophila types (see references in the introduction), but there is no way to predict one value from knowledge of the other; thus, these were treated as separate song parameters. The pulse amplitude measurements were attempts to quantify a song's loudness. This is difficult to measure reliably, and the units specified are arbitrary (see results); however, we were careful to keep the gain settings during all recordings constant.

Because of the low amplitude of song produced at low temperatures and by some mutants, a background scaling factor (bsf) equal to 1 was used (see Villella *et al.* 1997, for an explanation of this feature of the method). Only trains with four or more pulses were logged, and the following IPI cutoffs (minimum to maximum in msec) were used, depending on the temperature: 15° (40–105 msec); 17.5° (30–95 msec); 20° (25–90 msec); 22.5° (20–85 msec); 25° (15–80 msec); 27.5° (10–75 msec); 30° (10–70 msec). These cutoffs were decided based on preliminary analysis and previous work (*e.g.*, Wheeler *et al.* 1989; Villella and Hall 1996) showing that, at some upper-limit, a so-called IPI would, in reality, be an interbout-interval.

The recordings were carried out with the INSECTAVOX at the specified temperature, which, at the beginning of the recording, was usually 1° less than the nominal one indicated; and, at the end of the recording, was 0.5° higher. The room in which the recordings were done was adjusted to the desired temperature; however, the light inside the INSECTAVOX caused slight temperature increases during a recording session. For recordings performed at relatively high temperatures (27.5–30°), a water bath was used to keep the flies in the desired condition, and a heating fan was used to warm up the INSECTAVOX. Flies were acclimatized to the different temperatures for at least 30 min before recordings. Virgin females, 1 day old from the attached-*X* stock (indicated above), and 3–7-day-old males of the various genotypes were used for the recordings.

The number of pulses per minute shown in Table 5 was obtained from the results of the song analyses described above. The wing-extension time was measured with the aid of electric timers, and the logging of this behavior was performed observing the same videotape recordings used for song analysis (*cf.* Villella and Hall 1996). The Wing-Extension Index (WEI) represents the durations of such behavioral bouts divided by the total recording time. The number of pulses per minute of wing extension was obtained by dividing the number of pulses per min by the Wing-Extension Index for each fly.

Statistics: Statistical analyses were carried out using JMP software (Macintosh version 3.1; SAS Institute, Inc., Cary, NC) and according to Sokal and Rohlf (1995). Nonparametric tests (Wilcoxon/Kruskal-Wallis) were used for the statistical analysis of convulsion episodes (Table 1). The song parameters IPI and CPP shown in Figure 1 and the number of pulses per minute of wing extension shown in Table 5 were subjected to log transformation prior to analysis of variance to approximate normality in the distributions and homogeneity of variances. Comparisons to the wild type and *cac* controls in Tables 3 and 4, respectively, were carried out using Dunnett's method, whereas the multiple comparisons of Table 5 used the Tukey-Kramer method.

RESULTS

***cacophony* is a temperature-sensitive mutant:** When exposed to high temperatures (∼37°) *cac* flies show frequent convulsions and pronounced locomotor defects. This convulsion phenotype is characterized by flies turning upside-down or on their sides, shaking their legs for a few seconds, and then turning right-side up. The flies also curl their abdomen severely, either when on their backs or when walking, and twist their bodies at the same time. In addition, occasionally the *cac* adults will walk sideways, spin around on the same spot for a couple of seconds (apparently completely disoriented), leap across the chamber, or jump and tumble up and down out of control. There was no obvious sequence in the occurrence of these phenotypes. After long exposures to 37°, *cac* flies spend more and more time on their backs, shaking the legs until they seem to collapse. This typically requires more than 1 hr of heating for 1-day-old flies, but much less for older ones (*cf.* Table 1). As long as leg movement was still occurring, the mutant individuals usually recovered in a few minutes after transfer to room temperature (25°).

In tests involving exposure of 20-day-old *cac*-expressing flies to 37°, hemizygous mutant males and *cac*/*Df(1)RC29* females were observed (materials and methods explains why hemizygous mutant females were used, notwithstanding the male-limited song defects caused by *cac*). Over the course of 5 min at 37°, the former flies ($n = 11$) spent about half of this time period on their backs or sides, the latter ($n = 10$) ∼40% of the time. The mutant males curled their abdomens during ∼35% of the observation periods; the (hemizygous) mutant females, ∼20%. Each mutant type spun around in the chamber for ∼1% of the 5 min. These convulsions were not observed in similar tests of cac^+-bearing male adults ($n = 5$). Such normal flies will occasionally lie on their backs after falling from the ceiling of the heated chamber, but they right themselves within a couple of seconds. Moreover, curling of the abdomen was rarely observed, and only as a fleeting action in cac^+ males. When it occurred, it was less severe with no twisting of the body. The nbA^{EE171} and nbA^{H18} visual mutants ($n = 5$ males each), caused by mutations that map to the same locus as *cac*, behaved like cac^+ in these assays.

Table 1 shows a documented comparison of heat-induced convulsions among genotypes and ages (albeit only enumerating the percentages of time flies spent on their backs or sides). It is obvious that females carrying the *cac* mutation heterozygous with the *RC29* deletion spent far more time, at any age, on their backs or sides than females heterozygous for a wild-type derived *X*-chromosome and *RC29*, or that deletion and nbA^{EE171} or nbA^{H18}. There was also a strong age effect in these experiments. Older flies (Table 1) showed convulsions more readily and collapsed sooner after the temperature was raised. This, however, might simply reflect the normal decrease in high temperature tolerance with age (Ashburner 1989), as suggested by the data from 20-day-old *RC29*/+ flies (Table 1).

The convulsion phenotype of *cac* and its associated locomotor problems might be exploited in the future,

TABLE 1

Loss of body control in heated mutants

Age	Genotype	*n*	Percentage of time fly on its back or side	
			1–15 min	16–30 min
1 day	*RC29/+*	10	0.9 ± 0.2	1.2 ± 0.4
	cac/RC29	10	23.1 ± 1.9	25.0 ± 3.5
9 days	*RC29/+*	12	1.9 ± 0.4	3.4 ± 1.6
	cac/RC29	13	44.3 ± 2.3	57.9 ± 3.9
	$nbA^{EE171}/RC29$	9	0.8 ± 0.2	2.9 ± 1.7
	$nbA^{H18}/RC29$	8	1.4 ± 0.2	2.9 ± 0.9
20 days	*RC29/+*	12	5.8 ± 0.9	15.6 ± 3.5
	cac/RC29	14	47.1 ± 3.3	77.6 ± 4.6

Convulsions exhibited by cacophony at 37° are compared among different genotypes involving this genetic locus and among adults of different ages. The proportions of time (in percentages) that these flies spent on their backs or side when exposed to the high temperature were measured, with the adult animals placed in chambers within a prewarmed plastic (matting-cell) wheel resting on a hot-plate (see materials and methods). Two to three flies were observed during a given (timed) observation. Wilcoxon/Kruskal-Wallis nonparametric tests showed that the differences between genotypes within each age class (*e.g.*, *cac/RC29 vs.* the others for 9-day-old flies) and ages within each genotype at the two time intervals were significant in all cases. Significance levels were adjusted to 0.005, owing to experiment-wise error (*cf.* Sokal and Rohlf 1995).

for example, to isolate new *cac* alleles or suppressers using simple knock-out assays. The results in Table 2 shows how this might be done. Here, almost all of the *cac/RC29* flies were knocked out within 10 min when placed over a 46° hot-plate, whereas the same happened only to 8 out of 150 (5.3 %) flies of the other three genotypes: *RC29/+*, $nbA^{EE171}/RC29$, and $nbA^{H18}/RC29$.

The fact that *cac* is a TS convulsion mutant raises two questions. The first is whether temperature variation might have an effect on the song produced by *cac* that is different from its effects on wild-type flies (Shorey 1962; Ritchie and Kyriacou 1994). The second question is whether other *D. melanogaster* mutants causing TS paralysis and related phenomena show any alterations in the courtship song. These two questions are addressed below.

TABLE 2

***Knock-out* tests under extreme heating**

Genotype	*n*	No. knocked-out after 10 min
RC29/+	50	2
cac/RC29	50	49
$nbA^{EE171}/RC29$	50	2
$nbA^{H18}/RC29$	50	4

Complete loss of bodily function, occurring when the key mutants were exposed to more extreme heating than used in Table 1. Here, a knock-out assay was carried out by placing the chamber-containing plastic wheel (preloaded with 1–5-day-old flies from different genotypes) on a hot-plate at ~46° (see materials and methods). Eight flies (two of each genotype) were assayed in a given execution of these tests. The difference between genotypes in the proportion of knocked-out flies is highly significant ($\chi^2 = 158.29$, d.f. = 3, $P < 0.0001$).

Temperature effects on the *cacophony* courtship song: To examine the effects that temperature variation might have on the pulse song produced by *cac*, a song analysis of *cac* and wild-type flies was carried out at temperatures ranging from 15–30° in steps of 2.5°. (cac^+ males are here called wild-type ones, although in reality the relevant Canton-*S* stock had been outcrossed to an attached-*X* one.) Also included in this analysis was the mutant $para^{ts1}$, because a preliminary analysis had found it to have an effect on song at 25° (see below). The results are shown in Figure 1, A–D. Four pulse-song parameters were examined: amplitude of sound, IPI, CPP, and IPF. It is evident in these plots that temperature had a major effect on amplitude and IPI of all three genotypes, while it is far less clear in the case of CPP and IPF, even though the temperature effect is significant for the latter (see legend for Figure 1). Significant genotype differences were observed for amplitude, IPI, and CPP but not for IPF. The results also show the basic differences between *cac* and normal songs (*cf.* Kulkarni and Hall 1987), that is, higher amplitude and CPP, as well as longer IPIs in the former compared to the latter. IPFs were also found in a previous study to be similar between these two genotypes (Wheeler *et al.* 1989).

Although the overall trend observed for amplitude and IPI is similar for the three genotypes (as the temperature rises, there is an increase in the former and a decrease in the latter), differences were revealed in the way the various types of males reacted to temperature.

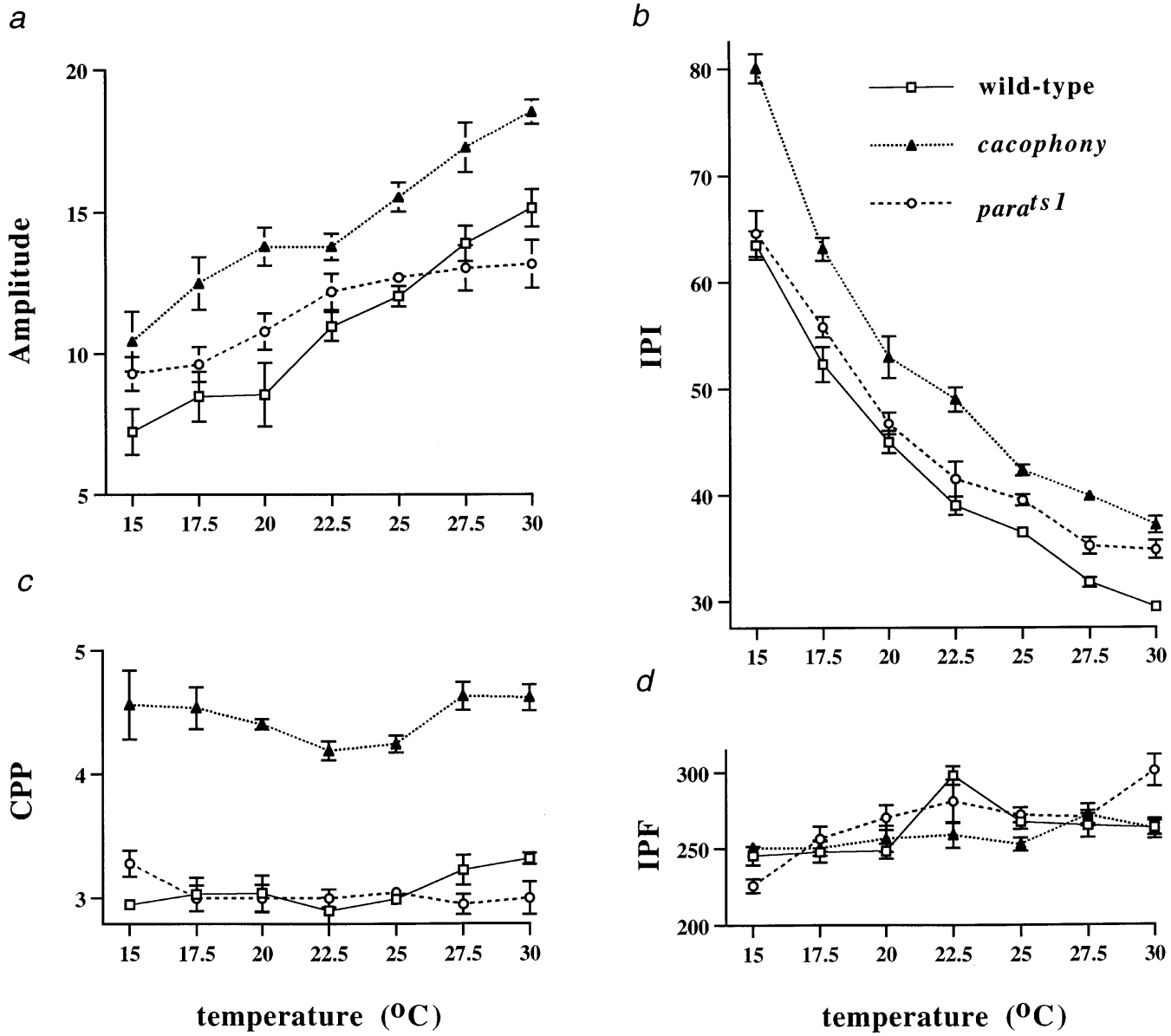

Figure 1.—Temperature-dependence of courtship-song characteristics exhibited by wild-type, *cacophony*, and *para*^{ts1} males. A minimum of 4 and a maximum of 16 flies were analyzed for each genotype and temperature. Four pulse-song parameters were measured: (A) amplitude of sound; (B) IPI (InterPulse-Interval); (C) CPP (Cycles-Per-Pulse); (D) IPF (Intrapulse-Frequency). Amplitudes were measured using an arbitrary scale, while IPIs and IPFs were measured, respectively, in milliseconds (msec) and Hertz (Hz). Means and their standard errors are plotted. Analysis of variance of all four song parameters was carried out, modeling temperature as a continuous variable. The analysis of the amplitude data indicated significant effects for genotype ($F_{[2, 140]} = 6.70$, $P = 0.0017$) and temperature ($F_{[1, 140]} = 35.37$, $P < 0.0001$), as well as for the genotype × temperature interaction ($F_{[2, 140]} = 6.03$, $P = 0.0031$). The same is true in the case of the IPI data, for which there are significant effects of genotype, ($F_{[2, 140]} = 7.70$, $P = 0.0007$), temperature ($F_{[1, 140]} = 1678.96$, $P < 0.0001$), and their interaction ($F_{[2, 140]} = 3.70$, $P = 0.0272$). Analysis of the CPP data showed significant effects for genotype ($F_{[2, 140]} = 22.13$, $P < 0.0001$) and the genotype × temperature interaction ($F_{[2, 140]} = 5.09$, $P = 0.0073$), but not for temperature ($F_{[1, 140]} = 0.86$, $P = 0.3543$); whereas, for the IPF data, the analysis indicated significant effects for temperature ($F_{[1, 140]} = 33.32$, $P < 0.0001$) and the genotype × temperature interaction ($F_{[2, 140]} = 4.50$, $P = 0.0127$), but not for genotype ($F_{[2, 140]} = 3.01$, $P < 0.0524$).

These differences are responsible for the significant genotype × temperature interactions observed (see legend to Figure 1). This is further illustrated in Figure 2, where the differences in IPI (Figure 2A) and amplitude (Figure 2B) between *cac* and wild type, and between *para*^{ts1} and wild type at each temperature, are plotted.

The difference in IPI between *cac* and wild type shows a significant negative correlation with temperature (see legend to Figure 2). The difference is actually larger at lower temperatures, a result that is somewhat counterintuitive if one considers that the convulsion phenotype of this mutant occurs at elevated tempera-

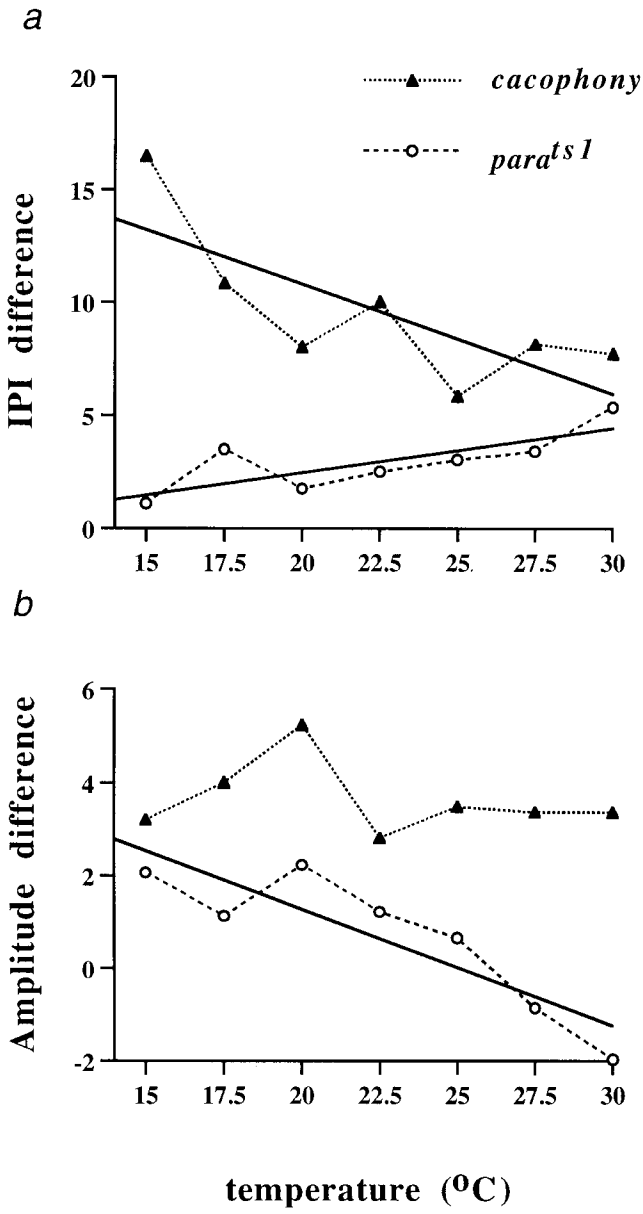

Figure 2.—Temperature-dependence of song IPI and amplitude. The courtship songs of the *cacophony* and *para*^{ts1} mutants were compared to those of (outcrossed) wild-type males. Significant correlations with recording temperature were found for the IPI differences (A) *cacophony* ($r = -0.7569$, $P = 0.0488$) and *para*^{ts1} ($r = 0.7761$, $P = 0.0402$), and for the amplitude differences (B) of *para*^{ts1} ($r = -0.8854$, $P = 0.0080$), but not for the amplitude differences of *cacophony* ($r = -0.1725$, $P = 0.7116$). The linear regression slopes are shown for the significant correlations.

tures. It is possible that this reflects in part the nonlinear nature of the IPI change with temperature. No significant correlation with temperature was observed for the amplitude differences.

The difference in IPI between *para*^{ts1} and wild type shows the opposite trend observed for *cac*. There is a significant positive correlation with temperature (see legend to Figure 2) with the larger IPI difference at 30°. In the case of amplitude, however, the differences between *para*^{ts1} and wild type show a significant negative correlation.

Song analysis of TS mutants: The fact that *cac* exhibits TS locomotor phenotypes prompted us to analyze the courtship song of certain TS mutants, most of which were isolated in *D. melanogaster* as general-locomotor mutants: *seizure* (*sei*^{ts1}), *temperature-induced-paralysis-E* (*tipE*), *paralytic* (*para*^{ts1}), *no-action-potential* (*nap*^{ts1}), *slowpoke* (alleles; *slo*^{1} and *slo*^{2}), and *cysteine-string-protein* (*csp*^{X1}) (see materials and methods for references, including one that reports the creation of the *csp*^{X1} mutant by reverse genetics).

The results of the song analysis of flies carrying the above mutations in homozygous and/or heterozygous condition are shown in Table 3. An extension of this analysis, an examination of a subset of these mutations in a *cac* background, is presented in Table 4. All recordings were done at 25°, and the same four pulse song parameters were measured: IPI, CPP, amplitude, and IPF.

As can be seen from the results of Table 3, *para*^{ts1} significantly increases the IPI at this temperature compared to the wild-type controls. This result led us to use this mutant in the analysis at different temperatures of the kind introduced above. Significant effects on singing were also observed for the two other mutations affecting sodium-channel function: lower amplitude and IPF in the case of *tipE*, and longer IPI for *nap*^{ts1}. However, the most interesting *nap*^{ts1} effect occurred when this mutation was in a *cac* background (see Table 4). While *nap*^{ts1} seems to enhance the IPI defects of *cac*, it had the opposite effect on cycles per pulse (CPP) and amplitude. For those parameters, *nap*^{ts1} seems to suppress some of the mutant nature of *cac*'s song. This interaction between *nap*^{ts1} and *cac* is illustrated in Figure 3, which shows some examples of song traces of males carrying the relevant genotypes. Males expressing (homozygous) *nap*^{ts1} alone generated trains of song sounds whose individual pulses not only looked liked those of wild type (not shown in Figure 3), but were also normal by analysis of intrapulse parameters (Table 3). The only numerical difference between *nap*^{ts1} songs and wild-type ones was a longer-than-normal interpulse interval (Table 3).

Attempts were made to record males homozygous for the *csp*^{X1} mutation of the *cysteine-string-protein* gene. However, these flies seemed too feeble to show any sign of courtship behavior. The same was true for the few *cac*; *csp*^{X1}/*csp*^{X1} males that were obtainable. In this respect, flies expressing either of these genotypes flies died within less than a week after adult-emergence. The heterozygous *csp*^{X1}/+ type gave increases in CPP and amplitude, as well as a decrease in IPF (Table 3). These effects were not significant in a *cac* background (*cac*; *csp*^{X1}/+ in Table 4), although the song-parameter changes were in the same direction as in *csp*^{X1}/+.

Mutations in two potassium-channel genes were examined. *sei*^{ts1} males showed no significant defects in

TABLE 3

Courtship song parameters from recordings made at a mild temperature

Genotype	*n*	IPI (msec)	CPP (no. cycles)	Amplitude (arbitrary units)	IPF (Hz)
wild-type	13	36.5 ± 0.4	3.0 ± 0.0	12.0 ± 0.4	267.3 ± 4.6
para^{ts1}	16	39.5 ± 0.5**	3.0 ± 0.0	12.7 ± 0.3	271.8 ± 4.9
tipE/tipE	5	36.4 ± 0.4	2.9 ± 0.1	8.3 ± 0.6**	241.8 ± 10.4*
nap^{ts1}/+	4	34.8 ± 0.6	3.1 ± 0.0	11.2 ± 0.4	251.0 ± 6.1
nap^{ts1}/nap^{ts1}	9	39.0 ± 0.7*	3.1 ± 0.1	11.1 ± 1.0	250.3 ± 6.3
csp^{X1}/+	7	35.4 ± 0.4	3.3 ± 0.1*	15.4 ± 0.5**	244.3 ± 4.7*
sei^{ts1}/sei^{ts1}	6	34.8 ± 0.4	3.1 ± 0.2	12.3 ± 0.8	250.0 ± 8.0
slo^{1}/+	4	36.3 ± 0.6	3.1 ± 0.0	11.1 ± 0.6	240.5 ± 4.5*
slo^{1}/slo^{1}	7	46.4 ± 0.8**	2.6 ± 0.1*	5.5 ± 0.3**	229.0 ± 3.0**
slo^{1}/slo^{2}	8	45.4 ± 0.6**	3.2 ± 0.1	8.4 ± 0.3**	247.9 ± 2.6
slo^{2}/slo^{2}	10	40.9 ± 0.8**	3.5 ± 0.1**	8.3 ± 0.4**	247.1 ± 4.8*
slo^{2}/+	12	36.1 ± 0.6	3.1 ± 0.1	11.5 ± 0.3	244.3 ± 3.4**

The results of computer-analyzing digitized courtship-song recordings, performed at 25°, are enumerated. The usual song parameters—IPI, CPP, amplitude, and IPF—were determined for genotypes indicated. As revealed in Figure 1, amplitudes were measured on an arbitrary scale, whereas IPIs and IPFs were measured in milliseconds (msec) and Hertz (Hz), respectively. Analysis of variance indicated significant differences between genotypes in all four parameters (IPI: $F_{[11, 89]} = 35.13$, $P < 0.001$; CPP: $F_{[11, 89]} = 8.31$, $P < 0.0001$; amplitude: $F_{[11, 89]} = 24.61$, $P < 0.0001$; IPF: $F_{[11, 89]} = 5.71$, $P < 0.0001$). Asterisks highlight genotypes that significantly differ from the wild-type control at 5% (*) and 1%(**) levels according to Dunnett's method (see materials and methods).

their songs. The mutant alleles, *slo^{1}* and *slo^{2}*, however, define *slowpoke* as a new courtship-song gene. The sounds produced by these two mutants were clearly aberrant in the pulse songs produced, and they were in fact often difficult to log due to the low-amplitude or polycyclic nature of pulses (at a given moment of singing: see below). Using the same criteria and IPI cutoffs used with the other mutants, all four song parameters examined are affected by these two *slo* alleles, which cause somewhat distinct song abnormalities. Males homozygous for the *slo^{1}* mutation produce very low-amplitude songs with long IPIs, and low CPP and IPF values. Isolated putative pulses, usually monocyclic signals, often occurred in *slo^{1}* song records; however, they were not logged because they did not occur in pulse trains (see materials and methods). In the case of the *slo^{2}* allele, the IPIs of homozygous mutant males were not as long, and the sound amplitude not as low, as in the case of *slo^{1}*. A train of pulses in the song produced by flies homozygous for *slo^{2}* often ends with a highly polycyclic pulse. In fact, the mean number of cycles per pulse of *slo^{2}/slo^{2}* flies is higher than the wild-type control (see Table 4). Isolated pulses were also often observed, but in this case (*cf. slo^{1}*) they are usually highly polycyclic.

TABLE 4

Courtship song parameters influenced by excitability mutations in a *cacophony* genetic background

Genotype	*n*	IPI (msec)	CPP (no. cycles)	Amplitude (arbitrary units)	IPF (Hz)
cacophony	13	42.3 ± 0.5	4.2 ± 0.1	15.5 ± 0.5	252.7 ± 4.1
cac; nap^{ts1}/+	9	41.0 ± 0.6	4.2 ± 0.1	16.0 ± 0.4	242.7 ± 4.0
cac; nap^{ts1}/nap^{ts1}	14	48.0 ± 0.8**	3.6 ± 0.1**	12.0 ± 0.6**	243.6 ± 3.7
cac; csp^{X1}/+	10	41.2 ± 0.7	4.4 ± 0.1	16.7 ± 0.4	247.1 ± 5.2
cac; slo^{1}/+	14	43.5 ± 0.5	4.2 ± 0.1	14.6 ± 0.5	244.6 ± 5.1
cac; slo^{1}/slo^{1}	5	50.4 ± 0.7**	4.0 ± 0.3	9.2 ± 0.6**	257.4 ± 9.8
cac; slo^{2}/+	10	42.8 ± 0.6	4.8 ± 0.1**	17.0 ± 0.5	265.0 ± 7.0

Recordings were performed for males of various genotypes, all of them including the *cacophony* mutation, at 25°. From the digitized versions of these records, the song parameters IPI (in msec), CPP, amplitude of the sounds, and IPF (in Hz) were determined. Analysis of variance indicates significant differences between genotypes for all four parameters (IPI: $F_{[6, 68]} = 24.71$, $P < 0.0001$; CPP: $F_{[6, 68]} = 13.58$, $P < 0.0001$; amplitude: $F_{[6, 68]} = 20.18$, $P < 0.0001$; IPF: $F_{[6, 68]} = 2.38$, $P = 0.0378$). Asterisks highlight genotypes that significantly differ from the *cacophony* control at 5% (*) and 1% (**) levels according to Dunnett's method (see materials and methods).

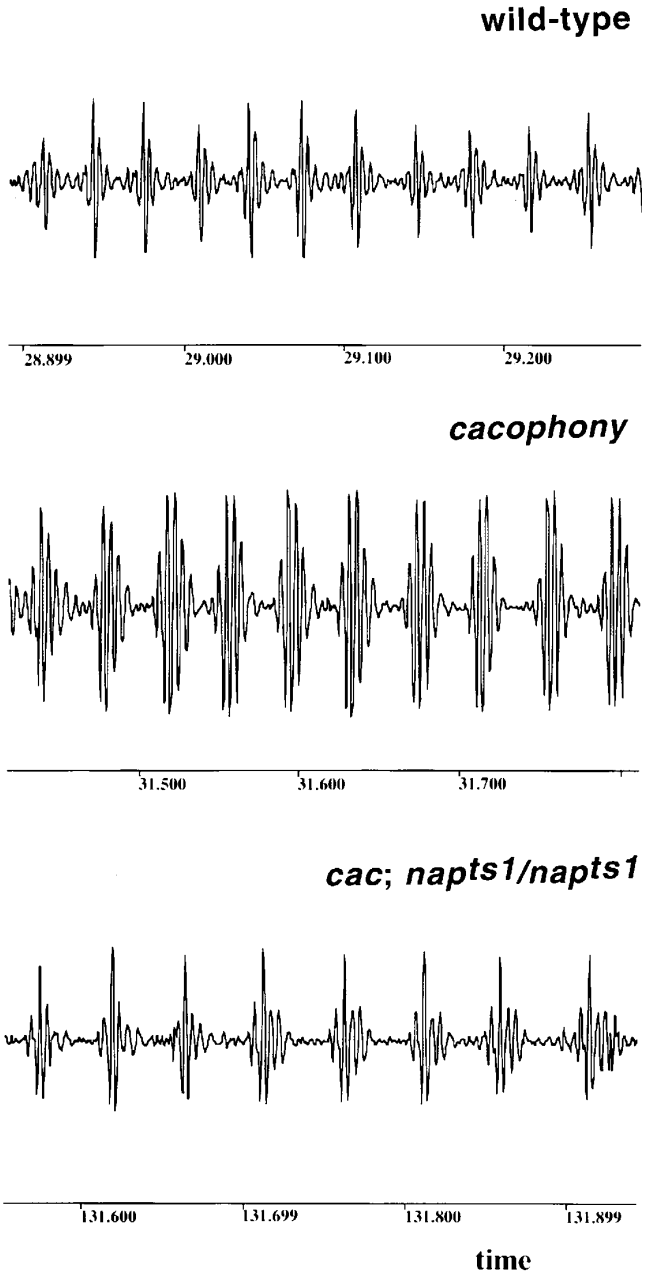

Figure 3.—Examples of song traces of wild-type, *cacophony*, and doubly mutant *cac* nap^{ts1} / nap^{ts1} males. How the nap^{ts1} mutation suppresses aspects of the polycyclic and high-amplitude nature of *cacophony*'s pulse-song, while elongating its IPI, is revealed in these pulse trains. The *time* base (abscissa) is in sec; these numbers vary considerably among the three lines of traces because they depict the singing behavior that occurred at different moments from the beginning of a given recording session.

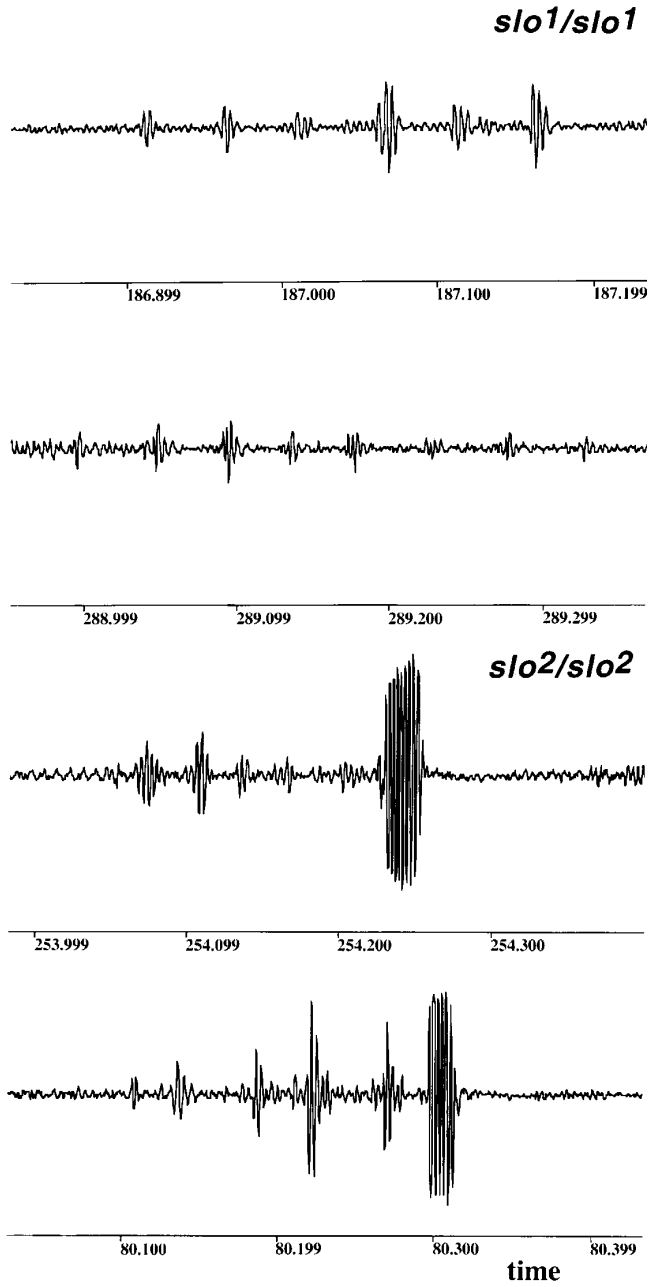

Figure 4.—Examples of pulse song recorded from males homozygous for the slo^1 and slo^2 mutations. Note the low amplitude and long InterPulse Intervals in the slo^1 / slo^1 traces. The types of pulse trains exemplified by the two slo^2 / slo^2 ones shown, which frequently included a polycyclic pulse at the end, were rarely seen in slo^1 recordings. Thus, slo^2 songs (notably, the bottom trace shown here) include pulse trains similar to those generated by *dissonance* mutant males (*cf.* Rendahl *et al.* 1996; Stanewsky *et al.* 1996).

Examples of song traces of these two mutants are shown in Figure 4. Heterozygous flies slo^1 / slo^2 show effects intermediate between the two homozygotes. The differences in the phenotypes between the two mutants obviously suggest differences in the molecular nature of the lesions that are unknown. slo^1 is a chemically induced mutation, while slo^2 was generated using gamma rays. Neither shows any gross chromosomal rearrangements (Atkinson *et al.* 1991; N. S. Atkinson, personal communication).

In a *cac* genetic background, the songs of the slo^1 / slo^1 flies also exhibited longer IPIs and lower amplitudes compared to the control (Table 4). Interestingly, the isolated pulses are polycyclic in this case, and the pulse

trains resemble the ones produced by *slo*^{2}/*slo*^{2} flies. Examples of song traces of this double mutant are shown in Figure 5. The aberrant nature of the sounds produced by flies carrying both mutations made it even more difficult to log their songs than for the single *slowpoke* mutants. In fact, trains with polycyclic pulses and IPIs longer than our standard cutoffs used were occasionally observed (see Figure 5). Flies homozygous for the *slo*^{2} allele rarely emerged as adults when the genetic background included *cac* (and no *cac*$^+$ allele). This could suggest some sort of interaction between the two genes, or just reflect the fact that *slo*^{2}/*slo*^{2} flies are quite sick (see materials and methods). The song of a single *cac*; *slo*^{2}/*slo*^{2} male was recorded and analyzed (IPI = 46 Hz, CPP = 5.4 Hz, amplitude = 11.0 Hz, and IPF = 275 Hz). These song-parameter values parallel the effects of this mutation in a *cac*$^+$ background, and the overall pattern resembles a more polycyclic version of *cac*; *slo*^{1}/*slo*^{1} songs. Note that in Table 5 the number of cycles-per-pulse in *cac*; *slo*^{2}/+ is significantly higher than the *cac* control, suggesting that, in this background, the *slo*^{2} mutation is not completely recessive.

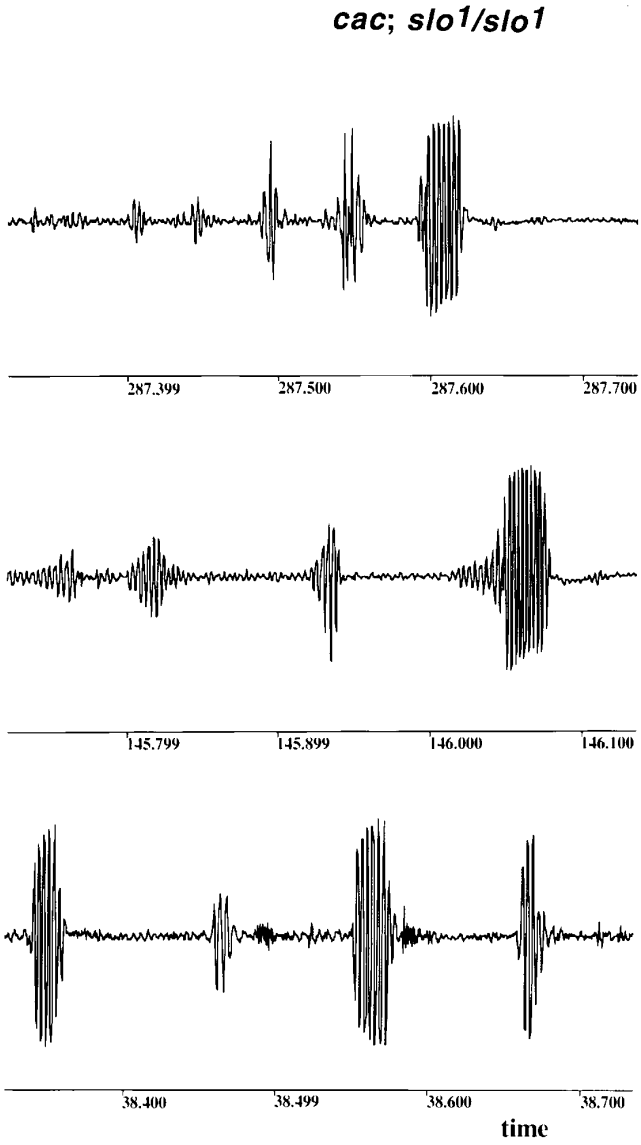

Figure 5.—Examples of pulse song from *slo*^{1}/*slo*^{1} males in a *cacophony* mutant background. The song trace on the top resembles the song of *slo*^{2}/*slo*^{2}, whereas the other two traces show polycyclic pulses; very long IPIs were also observed in these doubly mutant flies.

Finally, one effect that both *slo*^{1} and *slo*^{2} alleles shared is that flies carrying these mutations exhibited many courtship wing extensions without actually producing audible sound. To quantify this phenotype, the proportion of time flies extended their wings was logged and compared to the number of pulses per minute produced. As can be seen from the data in Table 5, the number of pulses per minute of wing extension is far lower in *slo*^{1}/*slo*^{1}, *slo*^{1}/*slo*^{2}, and *slo*^{2}/*slo*^{2} flies than in the case of *slo*^{1}/+ and *slo*^{2}/+ controls.

DISCUSSION

Much recent progress has been made in the genetic dissection of Drosophila's sexual behavior, as more and more genes are being discovered and characterized, including at the molecular level (Hall 1994a; Yamamoto *et al.* 1997). The identification and isolation of mutants that affect the courtship song production in *D. melanogaster* have two main goals. One is the dissection of the genetic and molecular components of the

TABLE 5

Rate of song pulse production affected by *slowpoke* mutations

Genotype	*n*	Pulses/min	WEI	Pulses/min of wing ext.
slo^1/+	4	345.9 ± 11.9	0.60 ± 0.03	577.2 ± 28.2
slo^1/*slo*^{1}	7	11.7 ± 9.8	0.37 ± 0.07	66.6 ± 19.7
slo^1/*slo*^{2}	8	31.3 ± 4.6	0.38 ± 0.05	86.1 ± 9.4
slo^2/*slo*^{2}	10	29.3 ± 3.7	0.31 ± 0.03	97.4 ± 11.0
slo^2/+	12	274.9 ± 22.5	0.58 ± 0.03	481.4 ± 33.8

The numbers of pulses per minute were obtained from the same song records that led to the other singing parameters shown in Table 3. The wing-extension time was measured by observing videotape recordings of the male-female interactions (whereby a separate track on the tape recorded the audio portion of the courtships). The Wing-Extension Index represents the amount of time during a given videotape record of the courtship, during which the male extended one of his wings or the other (near the female), divided by the total recording time. The number of pulses per minute of wing extension was obtained for each fly separately by dividing the number of pulses per min by the WEI. Analysis of variance revealed highly significant differences between genotypes ($F_{[4, 36]} = 47.25$, $P < 0.0001$). Tukey-Kramer multiple comparisons divided the genotypes in two groups: *slo*/+ and *slo*/+ forming one group and *slo*^{1}/*slo*^{1}, *slo*^{1}/*slo*^{2}, and *slo*^{2}/*slo*^{2} the other.

sound production machinery, as well as its neural control. The other is the identification and characterization of genes that might diverge in their structure and function over evolutionary time and be involved in the courtship-song differences that are so salient among species of this genus (*e.g.*, Ewing 1989).

Mutations in Drosophila ion channels are often associated with gross locomotor defects, including heat-induced paralysis (Wu and Ganetzky 1992), but some of these mutants exhibit subtle behavioral impairments, such as learning and olfactory defects (Cowan and Siegel 1986; Lilly *et al.* 1994). The finding that a song mutation *cacophony*, also shows a TS convulsion phenotype is therefore not surprising and is consistent with the likelihood that *cac* has suffered a mutation in a calcium channel gene (Smith *et al.* 1996; Peixoto *et al.* 1997). This kind of pleiotropy, which is further exemplified by the other visual and lethal mutations at the locus (Kulkarni and Hall 1987; Smith *et al.* 1996), is a common feature of behavioral genes (Hall 1994b). For example, the clock gene *period* (*per*) influences at least three different temporal aspects of Drosophila behavior and development (*e.g.*, Konopka and Benzer 1971; Kyriacou and Hall 1980; Kyriacou *et al.* 1990). Another example among song genes is *dissonance*, mutated at the *nonA* locus; the $nonA^{diss}$ mutant has both singing and visual problems (Kulkarni *et al.* 1988; Rendahl *et al.* 1992, 1996; Stanewsky *et al.* 1996). These pleiotropies have important implications for the evolution of such behavioral genes and the phenotypes they control (see below).

The song analysis of temperature-sensitive mutants identified at least one new song gene, *slowpoke*. Flies carrying mutant alleles at this locus present a number of problems in their singing. The song of one of the two alleles examined slo^{1} is characterized by very low-amplitude pulses and long IPIs. In the case of slo^{2}, the trains of pulses often end with a highly polycyclic pulse. This makes some of the singing bouts for slo^{2} males resemble those often produced by *dissonance* (Kulkarni *et al.* 1988; Rendahl *et al.* 1992, 1996; Stanewsky *et al.* 1996). Both *slowpoke* alleles performed many wing extensions that resulted in little or no song.

As introduced above, *cacophony* is one of the most interesting song mutations from an evolutionary point of view, at least in part because its abnormal pulses are nicely patterned, as in the case of wild-type males from various Drosophila species, and do not appear to be pathologically defective. A similar statement is possible about the songs of *slowpoke* males, although perhaps some of these mutant song bouts are more in the category of an erratic mess. Nevertheless, it is hard to believe that the song produced by double mutants *cac*; slo^{1}/slo^{1} comes from *D. melanogaster* males, so striking are the differences from the wild-type patterns.

The behavioral analysis revealed some additional candidates for song genes. Although some of these defects were subtle, the results obtained with $para^{ts1}$ and nap^{ts1} are potentially interesting. For example, the changes in the IPI × temperature and amplitude × temperature slopes obtained with $para^{ts1}$ (Figure 1) are connected with a possible evolutionary variation in Drosophila courtship that has not often been examined (however, see Ritchie and Gleason 1995). The phenotypic interaction observed between nap^{ts1} and *cac* not only points to the former as another song gene, but also suggests a possible interaction at the molecular level. It is possible that nap^{ts1} affects expression of the *X*-chromosomal *cacophony* gene in a manner that is analogous to *nap*'s (*mle*'s) interaction with the similarly located *para* gene (Wu and Ganetzky 1980; Ganetzky 1984; Kernan *et al.* 1991).

Another issue concerning the evolution of song genes is their molecular nature and the pleiotropy of neuronal-excitability mutants, which is one theme of this article. Might any neurological or behavioral mutant be so pleiotropic (*cf.* Hall 1994b) that this would include song defects? We think not because Kulkarni and Hall (1987) performed a survey of several such mutants and none was found to be song-defective. *tipE* males were deemed normal in the older study. The slight anomaly of this mutant's song indicated in Table 5 had to be teased out by a more detailed numerical analysis than was performed previously (Kulkarni and Hall 1987).

A fair fraction of the song mutants resulting from changes in genes that have been characterized at the molecular level involve membrane excitability. Not surprisingly, these basic functions, when mutated, lead to grossly appreciable defects in behavior. Only some of these mutants are song-defective as well (Kulkarni and Hall 1987, and the current article). *cacophony* now finds itself in this category, that is, the courtship variant mutant that started out as a song mutant but is now known to have other phenotypic defects, such as heat-induced convulsions. This kind of general impairment could be at least as detrimental to fitness as *cac*'s song abnormality.

Other pleiotropic song mutants with molecular correlates involve the regulation of gene expression (considered in general terms: transcription or RNA processing). In addition to the *period* and *dissonance* mutants in this category (as reviewed by Hall 1994a,b), consider the *fruitless* gene and its mutants. These courtship mutations defined a locus encoding a transcription factor (Ryner *et al.* 1996; Ito *et al.* 1996). *fru* mutations affect courtship song, as well as other aspects of the fly's reproductive behavior (Villella *et al.* 1997), including fertility. Pleiotropies of these sorts place important constraints on the evolution of these behavioral genes.

Genetic variation for features of the Drosophila courtship song have been reported from natural populations (*e.g.*, Ikeda *et al.* 1980; Kawanishi and Watanabe 1980; Ritchie and Kyriacou 1994; Ritchie *et*

al. 1994). It is possible that the level of genetic variability observed is influenced not only by sexual selection acting on the song parameters themselves, but also by selection on the pleiotropic effects of these putative song genes, which are likely to have broader biological significance than that. These pleiotropic effects could even include other aspects of the mate recognition system (Kaneshiro 1987). For example, there are *smell-blind* mutations at the *para* locus (Lilly *et al.* 1994), which affect the response of males to female pheromones (Tompkins *et al.* 1980; Gailey *et al.* 1986). It is also conceivable that directional selection acting on some of these pleiotropic effects, for example, selection for temperature tolerance and ion-channel genes, could drive changes in the song repertoire that could eventually lead to reproductive isolation between different populations.

While the constraints associated with pleiotropy certainly do not prevent the rapid evolution of Drosophila courtship songs (*e.g.*, Ritchie and Gleason 1995), it might explain why there is little evidence for genes with major effects in the song in crosses between closely related species (*e.g.*, Pugh and Ritchie 1996). It is likely that the lovesong differences between most such species are based on the cumulative effect of very mild and subtle changes in several genes, at least a handful of them involving, for example, interspecific variations at the *cac*, *slo*, and *mle* (*nap*) loci.

The major innovations in song production in the genus Drosophila seem to have occurred among Hawaiian flies (Hoy *et al.* 1988; Hoikkala *et al.* 1994) for which founder-effect models of speciation have been proposed (Carson and Templeton 1984). These include, for example, the idea of fixation of a mutation in a major locus, via genetic drift, followed by selection for modifiers on its deleterious effects. Pleiotropy and epistasis have major roles in these models. For instance, Palopoli and Wu (1994) revealed from detailed analysis of hybrid male sterility between two sibling species of Drosophila that epistasis between conspecific genes is a key component of this sexually related phenotype. Epistatic interactions among song genes, such as the one found between *cac* and nap^{ts1} within *D. melanogaster*, could also have important implications for sexual selection on the phenotypes they control and on their potential role in speciation (Wright 1982).

Because of the role acoustic signals such as the Drosophila's lovesong play in female receptivity, mating preferences, and sexual isolation between species (Bennet-Clark and Ewing 1970; Schilcher 1976; Kyriacou and Hall 1982, 1986; Greenacre *et al.* 1993; Tomaru *et al.* 1995), song factors are among the best candidates for the so-called "speciation genes" (Coyne 1992). The behavioral analysis we present here reveals that mutations in loci affecting ion-channel function might be a source of genetic variation in the fly's lovesong. Because of their enormous diversity (Hille 1992), channel genes might turn out to be among the most common classes of song genes.

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LITERATURE CITED

Ashburner, M., 1989 *Drosophila: A Laboratory Handbook.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Atkinson, N. S., G. A. Robertson and B. Ganetzky, 1991 A component of calcium-activated potassium channels encoded by the *Drosophila slo* locus. Science **253:** 551–555.

Bennet-Clark, H. C., and A. W. Ewing, 1970 The love song of the fruit fly. Sci. Amer. **223** (No. 1)**:** 84–92.

Bernstein, A. S., E. K. Neumann and J. C. Hall, 1992 Temporal analysis of tone pulses within the courtship songs of two sibling *Drosophila* species, their inter-specific hybrid, and behavioral mutants of *D. melanogaster.* J. Insect Behav. **5:** 15–36.

Carson, H. L., and H. L. Templeton, 1984 Genetic revolutions in relation to speciation phenomena: the founding of new populations. Annu. Rev. Ecol. Syst. **15:** 97–131.

Cowan, T. M., and R. W. Siegel, 1986 *Drosophila* mutations that alter ion conduction disrupt acquisition and retention of a conditioned odor avoidance response. J. Neurogenet. **3:** 187–201.

Cowling, D. E., and B. Burnet, 1981 Courtship songs and genetic control of their acoustic characteristics in sibling species of the *Drosophila melanogaster* subgroup. Anim. Behav. **29:** 924–935.

Coyne, J. A., 1992 Genetics and speciation. Nature **355:** 511–515.

Elkins, T., B. Ganetzky and C.-F. Wu, 1986 A *Drosophila* mutation that eliminates a calcium-dependent potassium current. Proc. Natl. Acad. USA **83:** 8415–8419.

Ewing, A. W., 1989 *Arthropod Bioacoustics.* Cornell University Press, Ithaca, NY.

Ewing, A. W., and J. A. Miyan, 1986 Sexual selection, sexual isolation and the evolution of song in the *Drosophila repleta* group of species. Anim. Behav. **34:** 421–429.

Feng, G., P. Deak, M. Chopra and L. M. Hall, 1995 Cloning and functional analysis of tipE, a novel membrane protein that enhances Drosophila *para* sodium-channel function. Cell **82:** 1001–1011.

Gailey, D. A., R. C. Lacaillade and J. C. Hall, 1986 Chemosensory elements of courtship in normal and mutant olfaction-deficient *Drosophila melanogaster.* Behav. Genet. **16:** 375–405.

Ganetzky, B., 1984 Genetic studies of membrane excitability in Drosophila: lethal interaction between two temperature-sensitive paralytic mutations. Genetics **108:** 897–911.

Gorczyca, M., and J. C. Hall, 1987 The INSECTAVOX, an integrated device for recording and amplifying courtship songs of Drosophila. Dros. Inf. Serv. **66:** 157–160.

Greenacre, M., M. G. Ritchie, C. B. Byrne and C. P. Kyriacou, 1993 Female song preference and the *period* gene of *Drosophila melanogaster.* Behav. Genet. **23:** 85–90.

Hall, J. C., 1994a The mating of a fly. Science **264:** 1702–1714.

Hall, J. C., 1994b Pleiotropy of behavioral genes, pp. 15–27 in *Flexibility and Constraint in Behavioral Systems,* edited by R. J. Greenspan and C. P. Kyriacou. John Wiley & Sons, Chichester, UK.

Hille, B., 1992 *Ionic Channels of Excitable Membranes.* Sinauer Associates, Sunderland, MA.

Hoikkala, A., and J. Lumme, 1987 The genetic basis of evolution of the male courtship sounds in the *Drosophila virilis* group. Evolution **41:** 827–845.

Hoikkala, A., K. Kaneshiro and R. R. Hoy, 1994 Courtship songs of the picture-winged *Drosophila planitibia* subgroup species. Anim. Behav. **47:** 1363–1374.

Hoy, R. R., A. Hoikkala and K. Kaneshiro, 1988 Hawaiian courtship songs: evolutionary innovation in communication signals of *Drosophila.* Science **240:** 217–219.

Ikeda, H., I. Takabatake and N. Sawada, 1980 Variation in courtship sounds among three geographical strains of *Drosophila mercatorum.* Behav. Genet. **10:** 361–375.

Ito, H., K. Fujitani, K. Usui, K. Shimizu-Nishikawa, S. Tanaka *et al.*, 1996 Sexual orientation in *Drosophila* is altered by the *satori* mutation in the sex-determination gene *fruitless* that encodes a zinc finger protein with a BTB domain. Proc. Natl. Acad. Sci. USA **93:** 9687–9692.

Kaneshiro, K. Y., 1987 The dynamics of sexual selection and its pleiotropic effects. Behav. Genet. **17:** 559–569.

Kawanishi, M., and T. K. Watanabe, 1980 Genetic variations of courtship song of *Drosophila melanogaster* and *D. simulans*. Jpn. J. Genet. **55:** 235–240.

Kernan, M. J., M. I. Kuroda, R. Kreber, B. S. Baker and B. Ganetzky, 1991 *nap*^{ts}, a mutation affecting sodium channel activity in Drosophila, is an allele of *mle*, a regulator of *X*-chromosome transcription. Cell **66:** 949–959.

Konopka, R. J., and S. Benzer, 1971 Clock mutants of *Drosophila melanogaster.* Proc. Natl. Acad. Sci. USA **68:** 2112–2116.

Kulkarni, S. J., and J. C. Hall, 1987 Behavioral and cytogenetic analysis of the *cacophony* courtship song mutant and interacting genetic variants in *Drosophila melanogaster.* Genetics **115:** 461–475.

Kulkarni, S. J., A. F. Steinlauf and J. C. Hall, 1988 The *dissonance* mutant of *Drosophila melanogaster:* isolation, behavior and cytogenetics. Genetics **118:** 267–285.

Kyriacou, C. P., and J. C. Hall, 1980 Circadian rhythm mutations in *Drosophila* affect short-term fluctuations in the male's courtship song. Proc. Natl. Acad. Sci. USA **77:** 6929–6933.

Kyriacou, C. P., and J. C. Hall, 1982 The function of courtship song rhythms in *Drosophila.* Anim. Behav. **30:** 784–801.

Kyriacou, C. P., and J. C. Hall, 1986 Inter-specific genetic control of courtship song production and reception in *Drosophila.* Science **232:** 494–497.

Kyriacou, C. P., M. Oldroyd, J. Wood, M. Sharp and M. Hill, 1990 Clock mutations alter developmental timing in *Drosophila.* Heredity **64:** 395–401.

Lilly, M., R. Kreber, B. Ganetzky and J. R. Carlson, 1994 Evidence that the Drosophila olfactory mutant *smellblind* defines a novel class of sodium-channel mutation. Genetics **136:** 1087–1096.

Lindsley, D. L., and G. G. Zimm, 1992 *The Genome of Drosophila melanogaster.* Academic Press, San Diego.

Loughney, K., R. Kreber and B. Ganetzky, 1989 Molecular analysis of the *para* locus, a sodium-channel gene in Drosophila. Cell **58:** 1143–1154.

Mastrogiacomo, A., S. M. Parsons, G. A. Zampighi, D. J. Jenden, J. A. Umbach *et al.*, 1994 Cystein string proteins: a potential link between synaptic vesicles and presynaptic Ca^{2+} channels. Science **263:** 981–982.

McCleskey, E. W., 1994 Calcium channels: cellular roles and molecular mechanisms. Curr. Opin. Neurobiol. **4:** 304–312.

Palopoli, M.F., and C.-I. Wu, 1994 Genetics of hybrid male sterility between Drosophila sibling species: a complex web of epistasis is revealed in inter-specific studies. Genetics **138:** 329–341.

Peixoto, A. A., L. A. Smith and J. C. Hall, 1997 Genomic organization and evolution of alternative exons in a Drosophila calcium-channel gene. Genetics **145:** 1003–1013.

Pugh, A. R. G., and M. G. Ritchie, 1996 Polygenic control of a mating signal in *Drosophila.* Heredity **77:** 378–382.

Rendahl, K. G., K. R. Jones, S. J. Kulkarni, S. H. Bagully and J. C. Hall, 1992 The *dissonance* mutation at the *no-on-transient-A* locus of *D. melanogaster*: genetic control of courtship and visual behaviors by a protein with putative RNA-binding motifs. J. Neurosci. **12:** 390–407.

Rendahl, K. G., N. Gaukhshteyn, D. Wheeler, T. A. Fry and J. C. Hall, 1996 Defects in courtship and vision caused by amino acid substitutions in a putative RNA-binding protein encoded by the *no-on-transient A* (*nonA*) gene of *Drosophila.* J. Neurosci. **16:** 1511–1522.

Ritchie, M. G., and C. P. Kyriacou, 1994 Genetic variability of courtship song in a population of *Drosophila melanogaster.* Anim. Behav. **48:** 425–434.

Ritchie, M. G., and J. M. Gleason, 1995 Rapid evolution of courtship song pattern in *Drosophila willistoni* sibling species. J. Evol. Biol. **8:** 463–479.

Ritchie, M. G., V. H. Yate and C. P. Kyriacou, 1994 Genetic variability of the inter-pulse interval of courtship song among some European populations of *Drosophila melanogaster.* Heredity **72:** 459–464.

Ryner, L. C., S. F. Goodwin, D. H. Castrillon, A. Anand, A. Villella *et al.*, 1996 Control of male sexual behavior and sexual orientation in Drosophila by the *fruitless* gene. Cell **87:** 1079–1089.

Schilcher, F. V., 1976 The role of auditory stimuli in the courtship of *Drosophila melanogaster.* Anim. Behav. **24:** 18–26.

Schilcher, F. V., 1977 A mutant which changes courtship song in *Drosophila melanogaster.* Behav. Genet. **7:** 251–259.

Shorey, H. H., 1962 Nature of the sound produced by *Drosophila melanogaster* during courtship. Nature **137:** 677–678.

Smith, L. A., X. J. Wang, A. A. Peixoto, E. K. Neumann, L. M. Hall *et al.*, 1996 A *Drosophila* calcium channel α-1 subunit gene maps to a genetic locus associated with behavioral and visual defects. J. Neurosci. **16:** 7868–7879.

Sokal, R. R., and F. J. Rohlf, 1995 *Biometry*, Ed. 3, W. H. Freeman & Co., New York.

Stanewsky, R., T. A. Fry, I. Reim, H. Saumweber and J. C. Hall, 1996 Bioassaying putative RNA-binding motifs in a protein encoded by a gene that influences courtship and visually mediated behavior in Drosophila: *in vitro* mutagenesis of *nonA.* Genetics **143:** 259–275.

Stea, A., T. W. Soong and T. P. Snutch, 1995 Voltage-gated calcium channels, pp 114–151 in *Ligand- and Voltage-gated Ion Channels*, edited by R. A. North. CRC Press, Inc., Boca Raton, FL.

Titus, S. A., J. W. Warmke and B. Ganetzky, 1997 The *Drosophila erg* K^+ channel polypeptide is encoded by the seizure locus. J. Neurosci. **17:** 875–881.

Tomaru, M., H. Matsubayashi and Y. Oguma, 1995 Heterospecific inter-pulse intervals of courtship song elicit female rejection in *Drosophila biauraria.* Anim. Behav. **50:** 905–914.

Tompkins, L., J. C. Hall and L. M. Hall, 1980 Courtship-stimulating volatile compounds from normal and mutant *Drosophila.* J. Insect Physiol. **26:** 689–697.

Villella, A., and J. C. Hall, 1996 Courtship anomalies caused by *doublesex* mutations in *Drosophila melanogaster.* Genetics **143:** 331–344.

Villella, A., D. A. Gailey, B. Berwald, S. Ohshima, P. T. Barnes *et al.*, 1997 Extended reproductive roles of the *fruitless* gene in *Drosophila melanogaster* revealed by behavioral analysis of new *fru* mutants. Genetics **147:** 1107–1130.

Wang, X.-J., E. R.. Reynolds, P. Deak and L. M. Hall, 1997 The *seizure* locus encodes the *Drosophila* homolog of the HERG potassium channel. J. Neurosci. **17:** 882–890.

Wheeler, D. A., S. J. Kulkarni, D. A. Gailey and J. C. Hall, 1989 Spectral analysis of courtship songs in behavioral mutants of *Drosophila melanogaster.* Behav. Genet. **19:** 503–528.

Wright, S., 1982 Character change, speciation and the higher taxa. Evolution **36:** 427–443.

Wu, C.-F., and B. Ganetzky, 1980 Genetic alteration of nerve membrane excitability in temperature-sensitive paralytic mutants of *Drosophila melanogaster.* Nature **286:** 814–816.

Wu, C.-F., and B. Ganetzky, 1992 Neurogenetic studies of ion channels in Drosophila, pp. 261–314 in *Ion Channels*, Vol. III, edited by T. Narahashi. Plenum Press, New York.

Yamamoto, D., J.-M. Jallon and A. Komatsu, 1997 Genetic dissection of sexual behavior in *Drosophila melanogaster.* Annu. Rev. Entomol. **42:** 551–585.

Zheng, W., G. Feng, D. Ren, D. F. Eberl, F. Hannan *et al.*, 1995 Cloning and characterization of a calcium channel α-1 subunit from *Drosophila melanogaster* with similarity to the rat brain type D isoform. J. Neurosci. **15:** 1132–1143.

Zinsmaier, K. E., K. K. Eberle, E. Buchner, N. Walter and S. Benzer, 1994 Paralysis and early death in cysteine string protein mutants of *Drosophila.* Science **263:** 977–980.

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